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Neuroprotective effects of olanzapine in a rat model of neurodevelopmental injury

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Abstract

Recent clinical studies have suggested that treatment with atypical antipsychotic drugs, such as olanzapine, may slow progressive changes in brain structure in patients with schizophrenia. To investigate the possible neural basis of this effect, we sought to determine whether treatment with olanzapine would inhibit the loss of hippocampal neurons associated with the administration of the excitotoxin, kainic acid, in neonatal rats. At post-natal day 7 (P7), rats were exposed to kainic acid via intracerebroventricular administration. Neuronal loss within the CA2 and CA3 subfields of the hippocampus and neurogenesis within the dentate gyrus of the hippocampus were then assessed at P14 by Fluoro-Jade B and BrdU labeling, respectively. Daily doses of olanzapine (2, 6, or 12 mg/day), haloperidol (1.2 mg/kg), melatonin (10 mg/kg), or saline were administered between P7 and P14. Melatonin is an anti-oxidant drug and was included in this study as a positive control, since it has been observed to have neuroprotective effects in a variety of animal models. The highest dose of olanzapine and melatonin, but not haloperidol, ameliorated the hippocampal neuronal loss triggered by kainic acid administration. However, drug administration did not have a significant effect on the rate of neurogenesis. These results suggest that olanzapine has neuroprotective effects in a rat model of neurodevelopmental insult, and may be relevant to the observed effects of atypical antipsychotic drugs on brain structure in patients with schizophrenia.
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1. Introduction

Nearly twenty years ago, it was hypothesized that the neuroanatomical abnormalities associated with schizophrenia might be the result of a static neurodevelopmental defect (Weinberger, 1987; Crow et al., 1989). However, it has also been reported that ventricular enlargement and volume loss in some brain structures is progressive during the course of the illness (Woods, 1998; DeLisi, 1999). In patients with childhood-onset schizophrenia, progressive losses of cortical gray matter have been observed (Thompson et al., 2001). Also, Pantelis et al. (2003), have reported progressive decreases in the volume of temporal lobe structures in young adult patients who are transitioning from having prodromal symptoms of schizophrenia to having the full form of the disorder.

Reports of progressive neuroanatomical abnormalities in subjects with schizophrenia suggest that neurodegeneration may occur during the pathogenesis of the disorder. If such observations are confirmed, treatments for schizophrenia should be assessed for their effects on neurodegenerative processes that may be relevant to schizophrenia. A variety of neurodegenerative mechanisms have been hypothesized to be involved in schizophrenia. Olney et al. have suggested that a developmental deficit of GABA interneurons could place an individual at later risk for neuronal injury via the uncontrolled activity of excitatory glutamatergic neurons (i.e., excitotoxicity) (Olney et al., 1999). Excitotoxicity is an attractive mechanism to explain neuronal injury in schizophrenia because it could be initiated and/or maintained through a variety of abnormally regulated neurotransmitter systems, including the monoamines (Farber et al., 1998) and acetylcholine (Olney et al., 1999). Also among

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such transmitter systems are the glucocorticoid stress hormones (Sapolsky, 2000), which could be triggered by environmental stressors, such as those (e.g., famine) that have been associated with an increased incidence of schizophrenia (Susser et al., 1998).

Another attractive theory to explain neuronal degeneration in schizophrenia is the inappropriate activation of apoptosis, a process normally associated with the elimination of redundant neurons during neurodevelopment (Johnson et al., 1995). Apoptosis occurs through a cascade of gene activation, which includes genes that both promote (i.e., BAX) (Schlesinger et al., 1997) and oppose (i.e., BCL-2) the process (Craig, 1995). In neonatal rats, we have shown that brief exposure to sublethal doses of the excitotoxin, kainic acid (KA), leads to a progressive loss of hippocampal neurons, and that apoptosis is the mechanism underlying this process (Montgomery et al., 1999). Also, the delayed neuronal loss triggered by KA administration at P7 is accompanied by an increase in neurogenesis, with some of the newly born neurons appearing to migrate to the pyramidal cell fields of the hippocampus (Dong et al., 2003). These results are consistent with observations previously made in adult animals exposed to KA (Bengzon et al., 1997). Thus, excitotoxic insults early in life, such as those that might be associated with hypoxia or other forms of perinatal trauma (Olney et al., 1999), could initiate a progressive process involving both the elimination and reorganization of neurons via apoptosis and neurogenesis.

The primary objective of this study was to determine whether treatment with olanzapine, a commonly used atypical antipsychotic drug, would inhibit the loss of hippocampal neurons and increase in hippocampal neurogenesis associated with the administration of the KA in neonatal rats (Dong et al., 2003). Olanzapine was selected for this study because of the results of a recent comparison of the long-term effects of olanzapine and haloperidol in patients with first-episode schizophrenia (Lieberman et al., 2005). In this study, treatment with haloperidol was associated with a greater rate of cortical gray matter loss than olanzapine, suggesting that the olanzapine might have had a special capacity to slow the neurodegeneration associated with schizophrenia.

2. Methods

2.1. Subjects

Sprague–Dawley dams with litters (8–12 pups per dam) were obtained from Harlan Bioproducts (Indianapolis, IN, USA). At post-natal day 7 (i.e., P7), pups were randomly assigned to one of six experimental groups. A total of 51 Spraque–Dawley rat pups were used in this study (6–10 animals per drug administration group). Procedures involving animals were conducted in accordance with institutional guidelines that are in compliance with national laws and policies.

2.2. Kainic acid administration

KA was administered to neonatal rats at P7 as previously described (Dong et al., 2003). Briefly, rat pups were taped to a surgical platform attached to a Kopf stereotaxic apparatus (Tujunga, CA), anesthetized with a 4% halothane/oxygen mixture, and maintained during surgery with a 1–4% halothane/oxygen mixture using a Fluotec atomizer (Fraser-Sweatman, Buffalo, NY). The scalp was swabbed with 10% betadine solution and 0.05 cc lidocaine was injected subcutaneously at the site of the incision. A 1 cm long incision was made exposing bregma. Then, a 25-gauge needle attached to a 1 μl Hamilton syringe was slowly lowered into the right ventricle using the following coordinates with respect to bregma: $A/P=-1.0$; $M/L=\pm 1.1$; $D/V=-3.3$. A total of 6.35 nmol KA (Sigma, St. Louis, MO) in 0.5 μl saline was injected into the ventricle at a rate of 0.1 μl every 2 min. The needle was then slowly raised and the procedure was repeated for the left ventricle. Afterwards, the needle was slowly withdrawn from the brain and the incision was sealed with a topical tissue adhesive. Animals were placed on a warm heating pad to recover and monitored until awake.

2.3. Drug treatment

Beginning on P8, 24 h after KA administration, animals were administered olanzapine at one of three doses (2, 6, or 12 mg/kg), haloperidol at one dose (1.2 mg/kg), the anti-oxidant, melatonin (10 mg/kg), or saline. The doses of olanzapine, and the single dose of haloperidol, were selected based on the results of prior studies in our laboratory in which the effects of these drugs were tested on locomotion in adult rats that had been administered KA (Bardgett et al., 2002). Melatonin has been previously observed to inhibit neurodegeneration related to both apoptosis (Pandey et al., 2003) and excitotoxicity (Chandrasekaran et al., 2004), and so was administered as a positive control. Beginning at P8, animals received i.p. injections of drug each day for six days. Because of olanzapine's short half life in rodents, the daily dose of olanzapine (as well as all other drugs to maintain consistency) was divided in half and administered twice daily. Haloperidol was prepared by dissolving 30 mg in 100 μl 20% acetic acid, and then diluting with saline to a final volume of 10 ml. Olanzapine was prepared by dissolving the 2, 6, or 12 mg/kg in 25, 50, or 150 μl of 20% acetic acid, respectively, and then diluting the solution with saline to a final volume of 2.5, 5, or 15 ml. Melatonin (10 mg/kg) was prepared by dissolving 5 mg of melatonin in 40 μl 100% ethanol and then diluting the solution with saline to achieve a final volume of 2 ml. Using these final solutions, the drugs were administered in a constant volume (2 ml/kg) to each animal. Control animals were administered the same volume (2 ml/kg) of saline.

2.4. Tissue preparation and confirmation of KA-induced neuronal loss

On P14, animals were deeply anesthetized with 60 mg/kg pentobarbital (i.p). Animals were then transcardially perfused for 2 min with 5 ml of 1% heparinized 0.01 M phosphate buffered saline (PBS) followed by 50 ml of 4% paraformaldehyde in 0.1 M PBS over 15–20 min. The brains were then removed and post-fixed at 4 °C for at least 24 h in 20% sucrose and 4% paraformaldehyde in 0.1 M PBS. The brains were then

embedded in Tissue-Tek embedding medium (Electron Microscopy Sciences, PA) and sectioned 35 μm thick at coronal plan using a cryostat.

To confirm the distribution of neuronal loss seven days after KA administration, Nissl staining was performed. Three coronal sections of the brain were selected in each animal to represent the anterior, intermediate, and posterior regions of the hippocampus and mounted to gelatin-coated slides. Slides were air-dried overnight. The slides were then rinsed by dipping in double distilled water ten times and then they were submerged in a 15% thionin solution for 30–60 s, until staining reached the desired intensity. The slides were then immersed in a graded series of ethanol for periods of 5 min in the following order: 50%, 70%, 90%, 100%, 100%, and 100%. Slides were then transferred to three successive baths of xylenes for periods of 5 min and coverslipped. The distribution of neuronal loss was then assessed using light microscopy.

2.5. Fluoro-Jade B labeling of degenerating neurons

Degenerating neurons were labeled on P14 with Fluoro-Jade B (FJB). FJB has been previously used to assess neurodegeneration following KA administration in rats and mice (Riba-Bosch and Perez-Clausell, 2004; Sriram et al., 2002; and Zucchini et al., 2002). FJB labels degenerating neurons, regardless of the cause of cellular degeneration (Schmued and Hopkins, 2000), possibly through its electrostatic attraction to polyamines, such as aminopropyl-butanediamine, diaminobutane, and diaminopentane, that are characteristic of degenerating neurons (Schmued and Hopkins, 2000). To assess FJB labeling, every 6th section beginning at the first hippocampal section was selected for staining. The sections were mounted on 2% pig skin gelatin-coated slides to increase adherence of the tissue to the slide during staining, and dried on a slide warmer for 1 h. The slides were then immersed in 100% ethanol for 3 min, followed by 70% ethanol for 2 min. The slides were then transferred into double distilled water for 2 min, 0.06% potassium permanganate for 15 min, rinsed in double distilled water for 2 min, and then labeled with a 0.0004% solution of FJB in 0.1 acetic acid for 30 min. The slides were rinsed afterwards with double distilled water and coverslipped with 75% glycerol in 0.01 M PBS. The sections were visualized under 465–495 nm excitation and the numbers of FJB-labeled neurons in the CA2/CA3 subfields were visually quantified in eight sections per animal. The CA2/CA3 subfield was selected for quantifying FJB-labeled neurons because these subfields show disproportionate cell loss following KA administration in neonatal rats (Humphrey et al., 2002). The boundary between CA1 and CA2/3 was primarily determined by evaluating the thickness of the pyramidal cell layer. The individual quantifying the number of FJB-labeled cells was blind to the drug that had been administered to the animal.

2.6. BrdU labeling of proliferating cells

Three doses of 5-bromodeoxyuridine (BrdU) were administered 24 h apart for three days (P11–13) prior to perfusion on P14. BrdU was prepared by dissolving 100 mg in 10 ml saline and then sonicating the solution at 37 °C for 40 min. Each animal received 50 mg/kg of BrdU (i.e., 5 ml/kg of the BrdU solution).

To quantify BrdU labeling, every 6th section beginning with the first section in which the hippocampus was visible was selected for staining (total of eight sections per animal). The free-floating sections were then rinsed 3 times for 5 min each with 0.01 M PBS. After denaturation of the DNA in 2 N HCL for 60 min at 37 °C, the sections were neutralized by rinsing them in 0.1 M borate buffer twice for 5 min. Next, the sections were rinsed with 0.01 M PBS three times for period of 5 min. The sections were then incubated in a blocking solution containing 5% normal horse serum and 0.3% triton in 0.01 M PBS for 1 h at room temperature. The sections were then incubated in anti-mouse BrdU primary antibody (1 : 800 in blocking solution; Boehringer-Mannheim, Indianapolis, IN) at 4 °C overnight. On the following day, the sections were rinsed three times for periods of 5 min with 0.01 M PBS and then incubated in horse anti-mouse secondary antibody (one drop in 20 ml blocking solution; Vector Laboratories) at room temperature for 2 h. The sections were then rinsed 3 times for periods of 5 min and incubated in avidin–biotin complex (Vector Laboratories) in 0.1 M PBS for 1 h at room temperature. The sections were then rinsed three times for periods of 5 min and BrdU-positive cells were visualized by incubating the sections with diaminobenzidine (DAB) solution (Vector Laboratories) for 10 min. Finally, the sections were rinsed three times with 0.01 M PBS, mounted to gelatin-coated slides, air-dried overnight, rinsed with double distilled water, and air-dried overnight again. After immersing the slides in xylenes three times for 5 min, they were coverslipped. BrdU labeling was visually quantified in the dentate gyrus of the hippocampal formation in eight sections per animal. The individual quantifying the number of BrdU-labeled cells was blind to the drug that had been administered to the animal.

To account for variability in the total numbers of neurons in tissue sections across animals, the densities of FJB- and BrdU-labeled neurons per total neuron number were measured and used in all data analyses. The density FJB- and BrdU-labeled neurons in the CA2/CA3 and dentate gyrus subfields (cells per mm^3) were calculated by multiplying the total number of cells counted by 10^4 and dividing by the total sample volume. The sample volume was determined by measuring the area using the C.A.S.T.-Grid software (Olympus, Denmark), which is included in the Olympus Stereologer program. To obtain a volume, the area was multiplied by the section thickness (35 μm).

2.7. Data analysis

Data from eight animals were not included in the analyses. In four of these animals (one saline-injected and one from each olanzapine group), neuronal loss within the hippocampal formation could not be identified. Four other animals died during the week following surgery. Two of these animals had been administered 6 mg/kg of olanzapine and two had been administered 2 mg/kg of olanzapine.

Data were analyzed using one-way ANOVAs to determine whether there was an overall effect of drug administration on the two dependent variables (densities of FJB- and BrdU-positive cells). Post hoc, between-group difference comparisons were made using Fisher's PSLD tests.

3. Results

Mean numbers of FJB- and BrdU-labeled cells in each of the experimental groups are given in Table 1. ANOVA revealed a significant effect of drug administration on the observed density of FJB-labeled cells [$F(537)=3.77$, $p=0.007$)]. Between-group comparisons revealed a statistically significant reduction in the density of FJB-labeled neurons in animals administered melatonin ($p=0.02$) and in animals administered the highest dose of olanzapine (12 mg/kg) ($p=0.01$). Reductions in FJB labeling (~50%) compared to saline controls were also seen in animals that had been administered the two lower doses of olanzapine, but these reductions did not attain significance (olanzapine, 2 mg/kg — $p=0.055$ and olanzapine, 6 mg/kg — $p=0.15$). The between-group comparison of haloperidol-administered animals and saline controls was not significant ($p=0.44$).

The ANOVA examining the effect of drug administration on the density of BrdU-positive cells was not significant ($F(537)=1.58$, $p=0.19$).

4. Discussion

The results of this study suggest that olanzapine at the highest dose tested ameliorated the hippocampal neuronal loss associated with intracerebraventricular KA administration in neonatal rats. Melatonin, but not the single dose of haloperidol tested, also ameliorated hippocampal neuronal loss in this animal model of neurodevelopmental injury. The neuroprotective effects of melatonin in this model were reassuring and support the validity of this animal model for detecting the neuroprotective actions of other drugs. Although increases in the density of BrdU-positive cells were noted in animals that had received both olanzapine and haloperidol, the effects of both antipsychotic drugs on the rate of cell proliferation in the hippocampus following intracerebroventricular KA administration was not significant.

Relatively little is known about whether drugs currently used to treat schizophrenia, including olanzapine, can inhibit neurodegeneration in animal models of excitotoxicity or apoptosis. Farber et al. (1996), showed that olanzapine, fluperlapine and clozapine blocked the neurogeneration associated with the administration of NMDA antagonists in rats. Similar results have been more recently reported by Wang et al. in neonatal rats administered repeated doses of phencyclidine (Wang et al., 2001). While the mechanism of such neuroprotective effects is not known, it may involve blockade of cholinergic receptors by these drugs, since the rat model of NMDA neurotoxicity has been linked to the excessive release of acetylcholine caused by a defect in GABA neurotransmission (Kim et al., 1999). Further, agonists at the nicotinic acetylcholine receptor have also been shown to have neuroprotective properties in rodent models of NMDA-induced neurotoxicity (Wenk et al., 1997), and in neuronal cultures deprived of trophic factors (Jonnala and Buccafusco, 2001; Jonnala et al., 2003). The effects of olanzapine in the present study would be consistent with such models in that olanzapine, but not haloperidol, is an antagonist at several muscarinic cholinergic receptors. Alternatively, olanzapine may also have had more direct effects on intracellular processes thought to be involved in apoptotic cell death. For example, olanzapine has recently been shown to up-regulate expression of superoxide dismutase and down-regulate the expression of the low affinity nerve growth factor p75 in PC12 cells (Li et al., 1999), and increases in SOD and decreases in p75 have previously been associated with reduced cell death in these cell culture systems.

Table 1

Drug administration	FJB-labeled cells* (Mean±SD)	BrdU-labeled cells* (Mean±SD)
Saline	8.1±33.5	69.4±42.0
Haloperidol (1.2 mg/kg)	4.7±33.2	132.6±104
Melatonin (10 mg/kg)	8.3±8.06	78.3±44.5
Olanzapine (2 mg/kg)	15.0±16.1	105.3±26.6
Olanzapine (6 mg/kg)	18.0±22.7	116.0±41.4
Olanzapine (12 mg/kg)	5.4±5.60	132.7±44.5

*Number of FJB- and BrdU-positive cells per $\mu m^3 \times 10^5$. See text for location of histological sections.

The results obtained in this study may be specific to the time points selected for KA administration and assessment of FJB and BrdU labeling. We chose to examine these cellular markers of neurodegeneration and neurogenesis at P14 because of our previous experiments administering KA to neonatal rats, which showed that the greatest changes in such markers at this time point (Dong et al., 2003). However, after KA administration to neonatal rats at P7, there is evidence of increased neurodegeneration and neurogenesis well into pubesence (Dong et al., 2003). Because mechanisms underlying apoptosis may differ in the neonatal and adult rat (Johnston et al., 2002), it is unclear from the present study whether olanzapine may have neuroprotective effects on the neurogeneration produced by KA (or other excitotoxins) at other age time points.

A limitation of this study includes the small sample size, which may have resulted in Type II error. To examine this possibility, a retrospective power analysis was performed. The sample size used only gave us weak power to detect a significant effect of drug administration on the rate of neurogenesis in the dentate gyrus of the hippocampus in all groups (power=0.48). The power to detect a significant effect on FJB labeling in the hippocampus in all groups was higher (power=0.90); therefore, the possibility of Type II error for this measure was less likely.

Another limitation of the current study includes the lack of sham lesion control. This would have allowed us to determine whether olanzapine's effects on neurodegeneration were specific to apoptosis induced by KA, or whether apoptosis related to normative developmental events was also affected. Also, in this study, we did not verify that FJB-labeled cells were degenerating via an apoptosis-related mechanism. However, in two previous studies of KA-induced hippocampal neurodegeneration, we confirmed the presence of apoptotic neurons in KA-

administered rats using morphological criteria at the light and electron microscope level (Dong et al., 2003). Finally, it should be kept in mind that olanzapine administration may have appeared to ameliorate neurodegeneration by affecting tissue quality, which might alter the penetration of dyes such as FJB. However, this would seem to be unlikely as similar decreases in BrdU labeling were not observed in animals administered olanzapine.

The results of this study should not be interpreted to suggest that olanzapine has a unique neuroprotective capacity. We only tested the effects of a single dose of the reference antipsychotic drug, haloperidol, and no other atypical or conventional antipsychotic drugs. Thus, it remains possible that other doses of haloperidol or other conventional or atypical antipsychotic drugs could have neuroprotective effects in this animal model similar to the effect we observed for the highest dose of olanzapine. It should also be noted that it is difficult to compare the dose of olanzapine observed to have neuroprotective effects in this animal model with the doses of olanzapine that are typically used to treat patients with schizophrenia. Nonetheless, the neuroprotective effect we observed with the relatively high dose of olanzapine used in this study (i.e., 12 mg/kg) suggests a hypothetical basis for the effects of olanzapine on the rate of cortical gray matter loss in patients with schizophrenia (Lieberman et al., 2005).

Recent clinical studies testing the effects of olanzapine on cognition and social function may be relevant to the question of whether olanzapine has neuroprotective effects in patients with schizophrenia. For example, in patients with schizophrenia, the capacity to halt progression or even reverse the cognitive deficits associated with schizophrenia (Green, 1996) may be indicative of a drug's capacity to inhibit neurodegeneration. In this regard, olanzapine has been shown to improve several cognitive deficits associated with schizophrenia (Purdon, 2000). Also, in a long-term controlled comparison of olanzapine and haloperidol, subjects treated with olanzapine showed gradual improvements in social performance and the capacity for work (Hamilton et al., 2000). However, most importantly, a recent comparison of the effects of olanzapine and haloperidol on brain structure in patients with first-episode psychosis suggested that treatment with olanzapine may slow the progressive reduction in cortical gray matter associated with the early stages of schizophrenia (Lieberman et al., 2005). While KA-induced hippocampal neurodegeneration is not a rodent model of schizophrenia per se, the effects of olanzapine observed in this study suggest an avenue of investigation to further investigate the neuroprotective effects of olanzapine and other antipsychotic drugs.

More research is needed to study the effects of conventional and atypical antipsychotic drugs in this and other animal models of neurodegeneration. These studies are needed to determine whether the effects of olanzapine observed in this study can be generalized to other models and other drugs. In addition, the effects of antipsychotic drugs could be investigated in neuronal culture models of cell death to allow for the detailed investigation of the underlying mechanisms associated with any neuroprotective effects. If clinical studies continue to support the hypothesis that treatment with at least some of the antipsychotic drugs can slow or even halt neurodegenerative processes that may be associated with schizophrenia, it will be critical to identify the mechanism by which such a benefit occurs. Identification of such a mechanism(s) could suggest entirely new strategies for the development of drug treatments for patients with schizophrenia.

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